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13. ABSTRACT (Maximum 200 Words)

We conducted a range-finding experiment to determine the appropriate dosage of androstenedione and Letrozole that will be used in the silastic implants. Next we evaluated effect of dietary genistein on the growth of estrogen-dependent breast tumors using an intratumoral aromatase expressing postmenopausal breast cancer (MCF-7Ca) model. For the range-finding experiment, ovariectomized athymic mice were divided into five treatment groups (15 mice per group); negative control (MCF-7Ca); 5 mg androstenedione (AD); 1 mg letrozole (LET), AD + LET, and 2 mg LET, AD + LET. For the interaction of genistein with Letrozole, ovariectomized athymic mice were divided into seven treatment groups (15 mice per group); negative control (MCF-7Ca); 5 mg androstenedione (AD); 1 mg letrozole (LET), AD + LET, AD + LET + 250 ppm GEN, AD + LET + 500 ppm GEN; AD + LET + 1000 ppm GEN. We identified an appropriate dosage of Letrozole and found that dietary genistein will stimulate the growth of MCF-7Ca tumors in the presences of Letrozole. Therefore, caution is necessary for postmenopausal women with estrogen-dependent breast cancer consuming dietary genistein.

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Genistein, Aromatase Inhibitors, Letrozole, Phytoestrogen, E-responsive breast cancer

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Dietary genistein negates the inhibitory effect of letrozole on the growth of aromatase-overexpressing estrogen-dependent human breast cancer (MCF-7Ca) cells implanted in ovariectomized athymic mice.

INTRODUCTION

Estrogens are synthesized by aromatization of androgen substrates via a series of reactions catalyzed by cytochrome P450 aromatase [1]. The highest levels of aromatase activity are present in the ovaries of premenopausal women and in the placenta of pregnant women. In postmenopausal women, ovarian production and circulating levels of estrogen declines and normal stromal and mammary epithelial synthesis of estrogens from C19 steroids by the cytochrome P450 enzyme, aromatase increases [2], and the major estrogen production sites are extragonadal sites, the adipose and skin fibroblasts [3]. In the adipose tissue and skin, the precursor is circulating AD derived from the adrenal cortex [4]. Approximately 60% of all breast cancer patients in the U.S. is estrogen-dependent breast cancer [5]. Pathological data showed an abnormal over-expression of aromatase in breast tumor [2, 6-11] and this aromatase production in breast tumors can significantly contribute to estradiol supply in tumors [12-14]. The effect of estrogens on the growth of breast cancers can be blocked by two manipulations: inhibition of estrogen action by anti-estrogens, which interact with estrogen receptors (ERs), and inhibition of estrogen synthesis by aromatase inhibitors. Aromatase inhibitor administration has been shown to be effective after, or instead of anti-estrogens [15]. Aromatase inhibitors could block the testosterone-stimulated tumor growth [10, 16]. Letrozole (Femara; Novartis Pharma AG, Basel, Switzerland), an imidazole derivative, is a potent, oral, nonsteroidal inhibitor of the enzyme aromatase. Isoflavones are known to inhibit aromatase effect, inhibit other enzymes involved in metabolism of steroid hormone, inhibit tyrosine kinases, and have other properties [17, 18]. Therefore, they are capable of modifying the estrogen level in women. Previously we demonstrated that dietary genistein stimulates the growth of estrogen-dependent human breast cancer (MCF-7) cells implanted in ovariectomized athymic mice [19]. We also showed that genistein negates the inhibitory effect of anti-estrogen, tamoxifen, on the growth of MCF-7 tumors [20]. Therefore, the elucidation of the effect of genistein on aromatase overexpressing estrogen-dependent breast cancer is a logical extension of our research on the effect of genistein. We evaluated the effect of dietary genistein on the growth of aromatase over-expressing estrogen-dependent human breast cancer.

BODY

Materials: Genistein was purchased from Indofine Chemical Company (Somerville, NJ), Androstenedione was purchased from Sigma Chemicals (St. Louis, MO). Eagle's minimum essential media (MEM, without gentamicin, with glutamine) and phenol red free MEM were purchased from Media Facility at University of Illinois at Urbana-Champaign. Bovine calf serum (BCS) was purchased from Hyclone (Logan, UT). Penicillin/streptomycin, trypsin/EDTA, were purchased from Invitrogen (Houston, TX). Reagents for real-time RT-PCR were purchased from Applied Biosystems (Foster City, CA), Synthegen (Houston, TX), Invitrogen (Carlsbad, CA), and Sigma (St. Louis, IN). Diet components were purchased from Dyets (Bethlehem, PA).

MCF-7Ca Cells: MCF-7Ca cells were provided from Dr. Santen (University of Virginia School of Medicine). MCF-7 Ca cells were generated by transfecting human aromatase gene into estrogen-dependent breast cancer (MCF-7) cells [21, 22]. MCF-7Ca cells were maintained in Eagles's minimum essential medium (MEM) with Eagle's salts with 1 mM pyruvate, 2 mM glutamine, 5% heat-inactivated BCS, 1% penicillin/streptomycin, and 600 mg/L neomycin.

Athymic Nude Mice. Ovariectomized athymic Balb/c (nude) mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for 1 week. Mice were ovariectomized at 21-day of age by the vendor and the allowed to recover for 7 days.

Androstenedione (AD) Pellet and Letrozole (LET) Silastic Implant. An AD pellet contained 5 mg of AD and 15 mg of cholesterol. A LET silastic implant (1.5 cm length, 0.1 cm inner diameter × 0.06 cm wall) contained either 1 or 2 mg of LET.

EXPERIMENTAL DESIGN

The experimental treatments used in the range-finding experiments are outlined in Table 1. The experimental dietary treatment for the study to evaluate the interaction of genistein and Letrozole are outlined in Table 2.

Table 1. AD pellet and LET implants

Group	AD pellet	LET implant	GEN	Mice (n)
MCF-7Ca				15
AD	5 mg			15
LET		1 mg		15
AD + LET	5 mg	1 mg		15
AD + LET	5 mg	2 mg		15

Table 2. AD pellet, LET implants, and dietary GEN

Group	AD pellet	LET implant	GEN	Mice (n)
MCF-7Ca				15
AD	5 mg			15
LET		1 mg		15
AD + LET	5 mg	1 mg		15
AD + LET + 250 GEN	5 mg	1 mg	250 ppm	15
AD + LET + 500 GEN	5 mg	1 mg	500 ppm	15
AD + LET + 1000 GEN	5 mg	1 mg	1000 ppm	15

Analysis of Tumor Growth: After one-week acclamation, mice were divided into ten groups (Table 1). AD pellets LET silastic tubes, or cholesterol implants were placed in mice. Three days after the AD and LET implantation, MCF-7Ca cells were harvested using 500 µl trypsin-EDTA (0.5% Trypsin, 5.3 mM EDTA•4Na)(Gibco-BRL, Grand Island, NY) per 100 mm culture plate. Cells were adjusted to 1×10^5 cells per 40 µl of Matrigel® (Collaborative Biomedical Products, Bedford, MA) and injected at 40 µl per site into each of the two flanks of the athymic mice. Then mice in the MCF-7Ca, AD, LET, and AD + LET groups were on isoflavone-free American Institute of Nutrition 93 growth semi-purified diet (AIN93G) [23]. Mice in the genistein treatment groups were fed AIN93G diet containing different dosages of genistein (250 ppm, 500 ppm or 1000 ppm). Soy oil was substituted with corn oil as a fat source to eliminate any additional components of soy being added to the diets. During the study, tumor growth and body weight were monitored weekly and food intake was measured throughout the study.

Statistics: Data from tumor surface area, were analyzed accordingly using one-way or repeated-measures analysis of variance according to the characteristics of the data set using the SAS program. If the overall treatment F-ratio was significant ($p < 0.05$), the differences between treatment means were tested with Fisher's Least Significant Differences test.

RESULTS

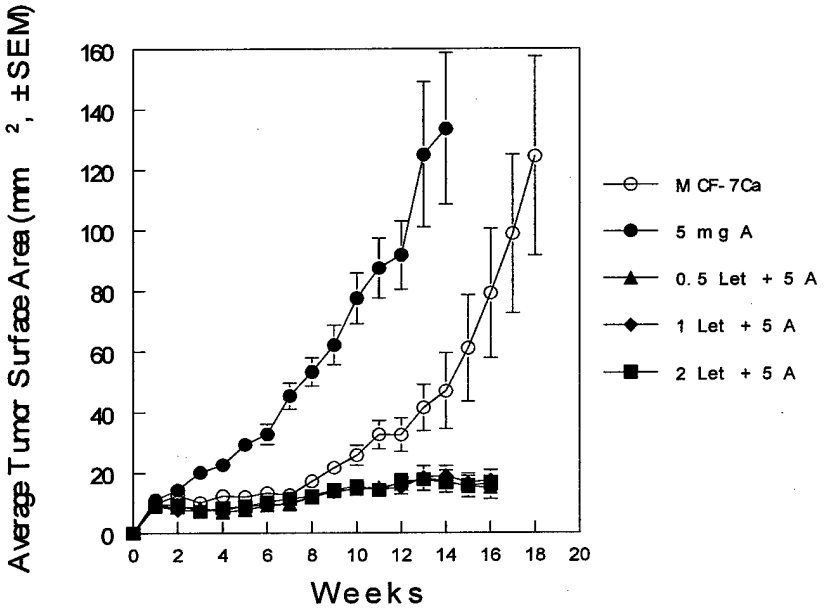

Figure 1. Range finding study to determine the appropriate dosage of Androstenedione and Letrozole.

Effect of Dietary Genitein on Growth of MCF-7Ca Tumors in the presence of AD and LET. At week 31, the average tumor size of the AD + LET + 1000 ppm reached 124.1 ± 21.0 mm^2, and the LET, AD + LET, AD + LET + GEN treatment groups were terminated. Average tumor areas were 2.6 ± 0.9 mm^2, 1.7 ± 0.7 mm^2, 56.7 ± 17.1 mm^2, 65.9 ± 13.3 mm^2, 124.1 ± 21.0 mm^2 for LET, AD + LET, AD + LET + 250 GEN, AD + LET + 500 GEN, AD + LET + 1000 GEN, respectively (Figure 1).

KEY RESEARCH

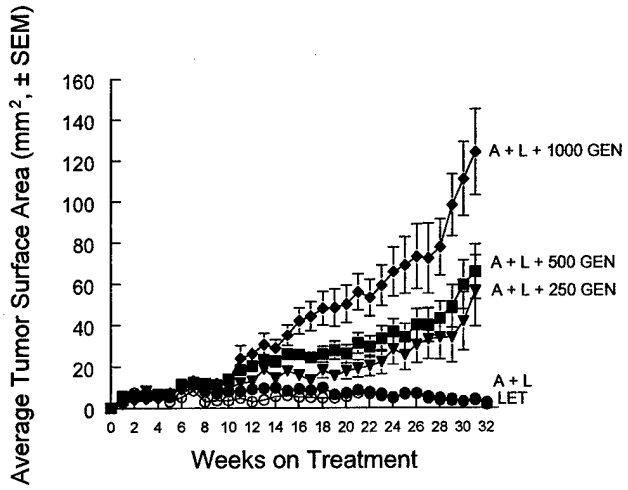

Figure 2. Effect of genistein on the growth of MCF-7Ca tumors in the presence of androstenedione and letrozole in athymic mice.

ACCOMPLISHMENTS:

-determined appropriate dosage of Letrozole
-observed that genistein can negate the inhibitory effect of Letrozole

REPORTABLE OUTCOMES:

Several related manuscripts have been published
PO1 form the NIA was applied for and awarded based on parallel research

CONCLUSIONS:

We have determined that an implant containing 5 mg of andorstenedione will greatly stimulate estrogen-dependent MCF-7Ca tumor growth and that 1 mg of Letrozole as a silastic implant will completely inhibit the stimulation. We have also observed that dietary genistein at dosages of 250, 500 and 1000 ppm can negate the inhibitory effect of Letrozole on androstenedione-stimulated MCF-7Ca tumor growth in vivo.

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APPENDIX

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Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of *R*- and *S*-equols and their differing binding and biological activity through estrogen receptors alpha and beta

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Abstract—Equol is a metabolite produced in vivo from the soy phytoestrogen daidzein by the action of gut microflora. It is known to be estrogenic, so human exposure to equol could have significant biological effects. Equol is a chiral molecule that can exist as the enantiomers *R*-equol and *S*-equol. To study the biological activity of racemic (±)-equol, as well as that of its pure enantiomers, we developed an efficient and convenient method to prepare (±)-equol from available isoflavanoid precursors. Furthermore, we optimized a method to separate the enantiomers of equol by chiral HPLC, and we studied for the first time, the activities of the enantiomers on the two estrogen receptors, ERα and ERβ. In binding assays, *S*-equol has a high binding affinity, preferential for ERβ (K_i[ERβ] = 16 nM; β/α = 13 fold), that is comparable to that of genistein (K_i[ERβ] = 6.7 nM; β/α = 16), whereas *R*-equol binds more weakly and with a preference for ERα (K_i[ERα] = 50 nM; β/α = 0.29). All equol isomers have higher affinity for both ERs than does the biosynthetic precursor daidzein. The availability and the in vitro characterization of the equol enantiomers should enable their biological effects to be studied in detail.

1. Introduction

Isoflavanoids found in soy, such as genistein and daidzein, have attracted great interest as dietary phytoestrogens that might be effective for menopausal hormone replacement therapy.[1] Suggestions have been made that these isoflavones might also be useful in the prevention or treatment of breast cancer.[2] Recent studies in animals, however, raise the possibility that the mammary cell growth-promoting effects of these compounds might, in fact, increase breast cancer risk and might also compromise the effectiveness of breast cancer hormone therapy using antiestrogens such as tamoxifen.[3] Thus, it is unclear whether the biological effect of these phytoestrogens will be a health benefit or a risk to humans. It is also possible that their potential for benefit to one tissue or one health condition could be compromised by their potential risk to another tissue or condition.

In examining the impact of the estrogenic activity of soy phytoestrogens, one needs to consider not only the isoflavones and their conjugates that are ingested, but also biologically active metabolites that might be generated from them in vivo. Daidzein, a prominent isoflavone in soy, is converted to the corresponding chroman, *S*-(−)-equol, a compound whose estrogenic activity exceeds that of daidzein.[4,5] This reductive metabolic conversion appears to result from the action of gut microflora.[6] Equol was first isolated from pregnant mares' urine in 1932[7] and was subsequently identified in the plasma of sheep (presumably derived from formononetin in red

Abbreviations: ER, Estrogen Receptor; HPLC, High Performance Liquid Chromatography; RBA, Relative Binding Affinity; HEC-1, Human Endometrial Carcinoma Cells-1.

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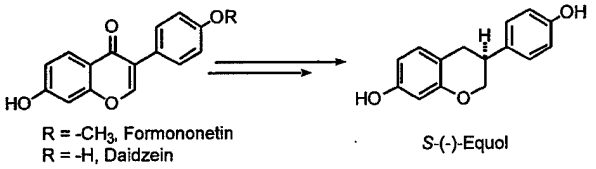
clover)[8] and in human urine (from daidzein).[9] Curiously, the extent of conversion of isoflavones to equol varies greatly among humans, presumably because of differences in the composition of gut microflora. Although typical laboratory animal species (mouse, rat, monkey) consistently produce high levels of equol, only about 35% of humans are high equol producers, and variations in urinary equol levels as great as 600-fold have been noted.[10–13]



R = -CH_3, Formononetin
R = -H, Daidzein

S-(-)-Equol

Equol is a chiral molecule that can exist in two enantiomeric forms, and the enantiomer produced by metabolic reduction from isoflavones is known to be *S*-(−)-equol. Equol is uterotrophic in mice, having a potency similar to that of genistein, and its binding affinity for the estrogen receptor from sheep uterus is reported to be about 1/250th that of estradiol.[14] (±)-Equol produced by synthesis has been shown to induce breast cancer cell proliferation in culture at concentrations as low as 100 nM.[15]

As part of an effort aimed at investigating the role that equol might play in breast cancer and the potential for differential activity of the two equol enantiomers through the two estrogen receptors, ERα and ERβ,[16] we encountered the need to prepare equol on a large scale and to obtain pure samples of *S*- and *R*-equol. In this report, we describe a convenient preparation of (±)-equol based on transfer hydrogenation that begins from readily available isoflavone precursors, as well as a biomimetic synthesis of (±)-equol. We also effect a robust chromatographic resolution of (±)-equol into *R*- and *S*-equol. We then evaluate, for the first time, the ERα and ERβ binding affinity and transcriptional activity of (±)-, *R*- and *S*-equol, and compare them with those of the biosynthetic precursor daidzein and the other abundant soy isoflavone genistein. Curiously, the equol enantiomers show differential behavior on the two ER subtypes, with *S*-equol having a preference for ERβ and *R*-equol having a preference for ERα.

2. Results

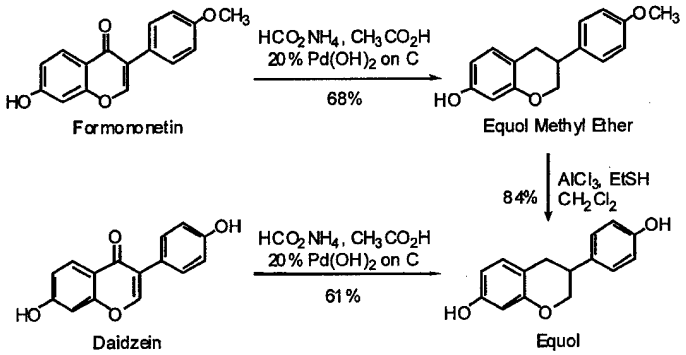
2.1. Synthesis

Our starting materials for the production of equol were the isoflavanoids formononetin and daidzein, which are readily available in large quantities from red clover and soy, respectively. The key step in the conversion of isoflavanoids to equol involves reduction of a vinylogous ester to an ether functional group. Most examples of such reductions involve either multiple steps or toxic reagents, with the exception of two reports,[17,18] both of which utilized hydrogenation in acetic acid with 10% palladium on carbon as catalyst. There are two major disadvantages with these particular procedures, however: first, the catalyst had to be activated prior to use by stirring under oxygen for 3 days and second, large amounts of catalyst were used. For example, 30 g of 10% palladium on carbon was used to reduce 6.7 g of daidzein diacetate. Given the cost of the palladium catalyst ($60 for 10 g), it is clear that scaling up the reduction to give, for example, 100 g of equol would be prohibitive.

Transfer hydrogenation is an attractive alternative to conventional hydrogenation, and a popular reagent for this reaction is ammonium formate.[19] Ram and Spicer noted that ketones and aldehydes undergo reduction to methylene groups using ammonium formate, provided that acetic acid is used as the solvent.[20] To test whether this methodology could be extended to the vinylogous ester carbonyl functionality of isoflavones, we performed exploratory experiments. Using formononetin in a model reaction, we found that with ammonium formate in acetic acid solvent and 10% palladium on carbon as the catalyst, we obtained monomethyl equol in 11% yield. The rest of the material consisted of products arising from partial reduction of either the double bond or the keto group. Longer reaction times did not lead to any improvement in yield. In further investigations in alternative solvents, such as tetrahydrofuran or ethanol, we observed only partial reduction of the double bond of isoflavanoids, as noted in the literature.[21] Attempts to use alternative transfer hydrogenation sources such as cyclohexene were also not successful. By varying the catalyst, we found that Raney Ni also led only to a mixture of partially reduced products. We were gratified to find, however, that Pearlman's catalyst,[22] which is 20% $Pd(OH)_2$ on carbon, was highly effective in this reduction.

Using this catalyst, we could reduce formononetin to monomethyl equol in 68% yield (Scheme 1). The methyl protecting group of the latter compound could be subsequently removed using aluminum trichloride in the presence of ethane thiol[23] to give equol in 84% yield. The overall yield for this two-step process is 57%. It is more convenient, however, to prepare equol directly from daidzein, and this direct reduction can be done in 61% yield (Scheme 1). We have used the latter one-step process to obtain >100 g of (±)-equol from daidzein.

In addition to methods involving hydrogenation, we also examined the possibility of using reagents such as $NaBH_4/CH_3CO_2H$ or Et_3SiH/CF_3CO_2H to reduce formononetin or its protected derivatives. But all such attempts led only to complete recovery of the starting material. Since these reagents are known not to reduce ester groups, the negative results presumably indicate that the carbonyl group of formononetin behaves more like an ester carbonyl rather than a ketone functionality. Thus, we anticipated that more reactive hydride donors, such as those derived from dihydroaromatics, could be used to accomplish the reduction. A good example of this class of hydride donors is dihydroacridine, a compound that has, in fact, been reported to reduce



Scheme 1.

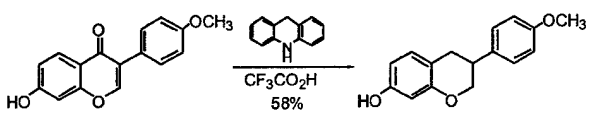
xanthones, which bear structural and functional similarities to isoflavones.[24] In our attempts to extend this reaction to isoflavones, we found that using trifluoroacetic acid as the solvent and dihydroacridine[25] as the reductant, formononetin could indeed be converted to monomethyl equol (Scheme 2), in moderate yield.

We believe that the above conversion is 'biomimetic' in nature, since dihydroacridine closely mimics the function of NADH, which is the most likely reducing agent in a biosynthetic context. An interesting sidelight of this reaction is that its progress can be followed by the increase in intense fluorescence of acridine as it is being generated.

2.2. Chromatographic resolution of *R*- and *S*-equol and stereochemical assignment

Equol isomers were initially separated using an analytical scale (2 mm diameter) β-cyclodextrin stationary phase liquid chromatography (LC) column under reversed phase conditions. Figure 1 shows the excellent separation of the two enantiomeric forms of racemic equol under these analytical scale conditions. The separation was then scaled up to a 10 mm diameter LC column to effect a semi-preparative isolation of approximately 10 mg total from each peak; good separations were obtained with injection of up to 400 μg of the racemate.

The individual collected fractions were analyzed using HPLC with electrospray mass spectrometric (ES–MS/MS) detection in the product ion mode (*m/z* 80–250 from *m/z* 243), and the mass spectra of the individual enantiomers were identical to that observed for racemic equol (product ions *m/z* 133, 123, 107, 105; data not shown). The purified fractions contained <0.3% of the other isomer, based on LC-ES/MS/MS analysis; furthermore, no other chemical contaminants were observed in either fraction by using LC-UV or LC-ES/MS/MS analysis.



Scheme 2.

The CD spectra obtained from the isolated peaks are shown in Figure 2. Peak 1 (17.5 min retention time) showed negative ellipticity at 280 nm, whereas Peak 2 (19.4 min retention time) showed positive ellipticity, with the two CD spectra being essentially mirror images of one another, properties that are characteristic of an enantiomeric pair. Based on prior structural assignment of (−)-equol as *S*-equol (Beilstein registry number 87752), Peak 1 in Figure 1 is *S*-(−)-equol and Peak 2 is *R*-(+)-equol. As noted above, *S*-(−)-equol is the naturally occurring enantiomer.[26,27]

2.3. Estrogen receptor binding affinity of R- and S-equol and the related soy isoflavones, daidzein and genistein

The binding affinities of (±)-, *R*- and *S*-equols to ERα and ERβ are shown in the first two sections of Table 1 (RBA values and K_i values), together with those of daidzein and genistein for comparison. Binding affinities were determined by a competitive radiometric binding assay using purified full length human ERα and ERβ, with [^{3}H]estradiol as tracer. In this assay, the affinities are obtained as Relative Binding Affinity (RBA) values, where estradiol has an affinity of 100.

While RBA values can be used to make comparisons between the binding affinities of different compounds on the *same* ER subtype, they are not appropriate for comparisons between *different* subtypes (e.g., ERα and ERβ), because estradiol (the reference compound and tracer in the competitive binding assays) binds to ERα with an affinity ca. 2.5 times higher than to ERβ (K_d = 0.2 nM for ERα versus K_d = 0.5 nM for ER).[28] Therefore, for each compound on each ER subtype, we have calculated the corresponding equilibrium binding inhibition constant or K_i values. The K_i values of the compounds represent their absolute affinities for both ER subtypes, and they are also the appropriate indices for comparison with the potencies of these compound (EC_{50} values) determined in the transcription assays (see below).

The equol enantiomers have distinctly different binding affinities: The absolute binding affinities (K_i values) of the naturally occurring equol enantiomer (*S*-equol) and of the unnatural enantiomer (*R*-equol) on ERα and ERβ are nearly reversed from one another, with *S*- equol having a strong preference for ERβ (β/α = 13) and

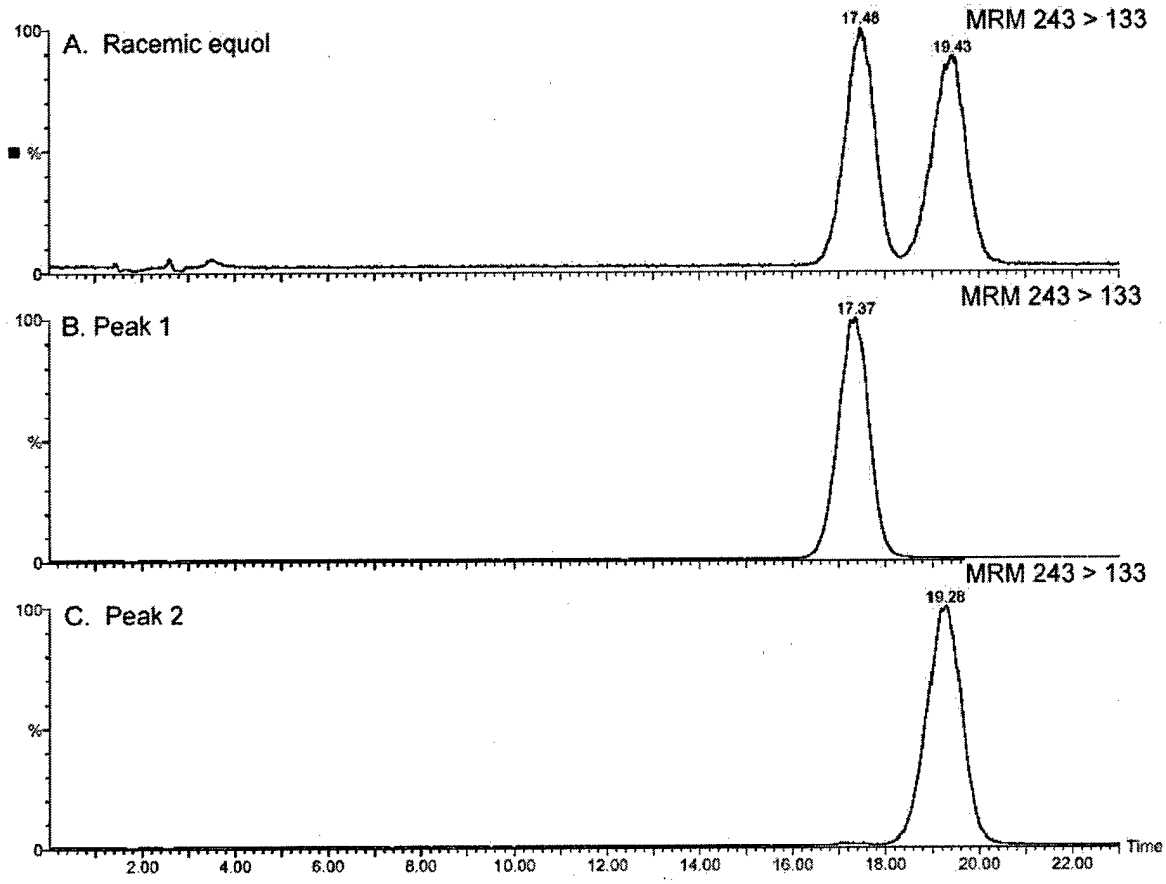


Figure 1. Separation of equol enantiomers by chiral phase liquid chromatography. Separations were done on an analytical column with a β-cyclodextrin-based chiral stationary phase, under reversed phase conditions (see Experimental Methods). *Panel A*. Chromatogram of equol racemate. *Panel B*. Chromatogram of *S*-(−)-equol obtained by preparative chiral LC. *Panel C*. Chromatogram of *R*-(+)-equol obtained by preparative chiral LC.

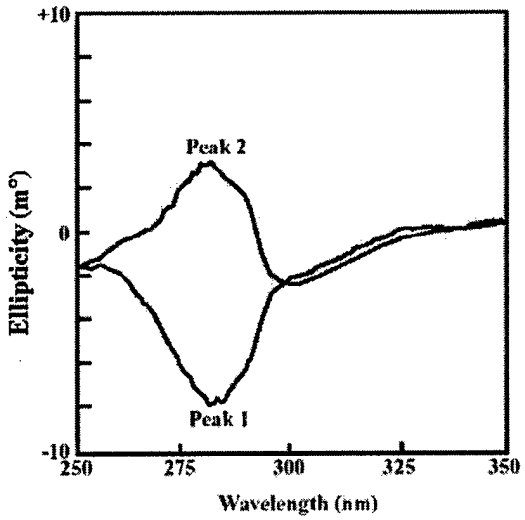


Figure 2. Circular dichroism spectra of equol enantiomers. The curve showing the negative ellipticity is from material in the first peak to elute (corresponding to *S*-(−)-equol, see Fig. 1A), and the curve showing the positive ellipticity is from material in the second peak to elute (corresponding to *R*-(+)-equol, see Fig. 1A). For details, see Experimental Methods.

R-equol having a moderate preference for ERα (β/α = 0.29). Significantly, the binding characteristics of *S*-equol, particularly its high ERβ preference, are most comparable to those of the most estrogenic soy isoflavone genistein, although *R*-equol binds better to ERα than does genistein. Also of note is the fact that all of the equols have much higher affinities for both ERs than does the abundant soy isoflavone daidzein, the biosynthetic precursor. As expected, the binding affinity of the racemate, (±)-equol, is almost exactly the average of that of the individual equol enantiomers. The much higher binding affinity of genistein relative to daidzein on ERβ is likely due to the decrease in polarity of the vinylogous ester functionality that occurs as a result of the intramolecular hydrogen bond between the carbonyl group and the proximal –OH function in genistein, an interaction that is absent in daidzein.[29]

2.4. Transcriptional activities of the equols and isoflavone compounds

The equols and the related isoflavones were assayed for transcriptional activity through both estrogen receptor subtypes. These cotransfection assays were conducted in

human endometrial carcinoma (HEC-1) cells, using expression plasmids for either full-length human ERα or ERβ and an estrogen-responsive luciferase reporter gene.[30] In all cases, transcriptional activity of the various compounds through either ERα or ERβ is presented relative to that obtained with 10^{-9} M estradiol, which is set at 100%. The dose–response curves are shown in Figure 3, and the EC_{50} values (which are comparable to the K_i values for binding affinity, not the RBA values) are summarized in the rightmost portion of Table 1.

From the transcription activation curves, it is apparent that all of the compounds are agonists on both ERα and

Table 1. Binding affinities[a,b] and Transcriptional potencies[c] of isoflavones and equol for ERα and ERβ

Ligand	RBA[a](%)		β/α[d]	K_i[b] (nM)		β/α[d]	EC_{50}[c] (nM)		β/α[d]
	hERα	hERβ		hERα	hERβ		hERα	hERβ	
Estradiol	100	100	1	0.2	0.5	0.4	0.021	0.11	0.19
Daidzein	0.010±0.006	0.040±0.001	4	2000	1300	1.5	250	100	2.5
Genistein	0.017±0.003	7.4±0.5	440	1200	6.7	180	80	6.6	12
(±)Equol	0.20±0.02	1.60±0.04	8	100	31	3.2	200	74	2.7
R(+)Equol	0.40±0.04	0.30±0.02	0.7	50	170	0.29	66	330	0.20
S(−)Equol	0.10±0.01	3.20±0.06	32	200	16	13	85	65	1.3

[a] RBA = Relative Binding Affinity (as a%) measured in a competitive binding assay (see Experimental). The K_d values of estradiol for ERα and ERβ are 0.2 nM and 0.5 nM, respectively.[28]

[b] K_i = equilibrium binding competition constant, calculated from the K_d of estradiol on ERα or ERβ: $(K_d/RBA)\times 100$.

[c] Transcriptional activity measured using a cotransfection assay in HEC-1 cells (see Experimental and Fig. 3). Transcriptional potency = EC_{50}.

[d] For each index, the β/α ratio is calculated such that the ratio is >1 for compounds having higher affinity or greater potency on ERβ than on ERα.

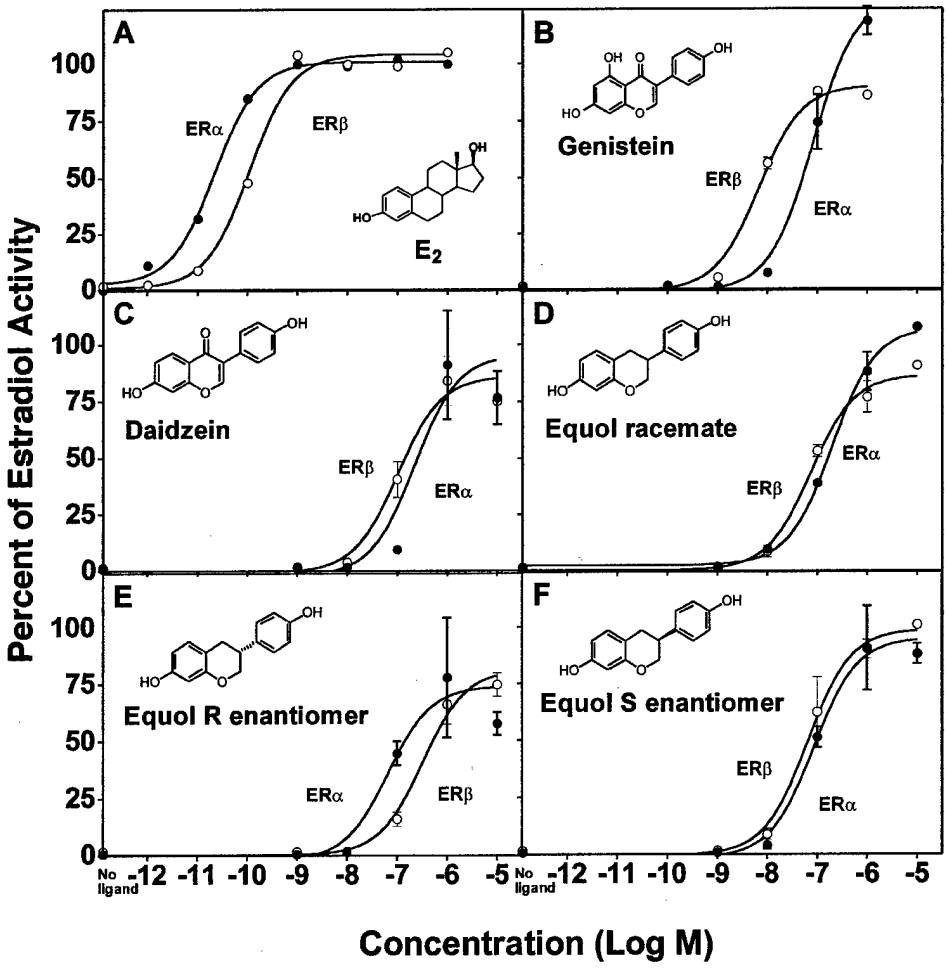


Figure 3. Dose–response curve for transcriptional activation by the indicated compounds. In each case, the solid circles are for ERα and the solid triangles are for ERβ. (**A**) estradiol (E_2); (**B**) genistein; (**C**) daidzein; (**D**) (±)-equol; (**E**) *S*-equol; (**F**) *R*-equol. Human endometrial carcinoma (HEC-1) cells were transfected with expression vectors for ERα or ERβ and an $(ERE)_2$-pS2-luc reporter gene and were treated with estradiol or the specified compound for 24 h, at the concentrations indicated. Luciferase activity was expressed relative to β-galactosidase activity from an internal control plasmid. The maximal activity with 1 nM estradiol was set at 100% (**A**). Values are the mean±SD from three separate experiments.

ERβ, giving maximal efficacies that are comparable to that of estradiol. Genistein appears to be somewhat of a superagonist on ERα, a characteristic that has been noted by others,[31] and the maximum efficacy of *R*-equol might be slightly less than that of estradiol.

When the potencies are compared, it is evident that all of the compounds are less potent than estradiol. Overall, the potencies (EC_{50} values) of the equols and the two isoflavones are comparable (ca. within a factor of 10) to their binding affinities (K_i values); the same is true for their ERα versus ERβ potencies (β/α values). The only significant difference between affinity and potency is that *S*-equol has a high binding affinity preference for ERβ (β/α = 13), but essentially no ER subtype preference in terms of transcriptional potency (β/α = 1.3). This is in contrast to the ERα preference of *R*-equol and the ERβ preference of genistein, evident in their binding affinities (β/α = 0.29 and 180, respectively), that are largely maintained in their transcriptional potencies (β/α = 0.20 and 12, respectively).

3. Discussion

Legumes (including soy, sprouts, and red clover) are rich sources of isoflavones that have been shown to be cancer-preventive.[32,33] The major isoflavones found in soybeans and soy products are genistin and daidzin (β-glycosides), and their aglycone forms, genistein and daidzein. Biochanin A and formononetin are proestrogenic isoflavones also found in legumes.[34] After ingestion, the β-glycosides undergo further metabolism by intestinal microflora,[35,36] and through the action of bacterial glucosidases, they are converted to aglycones (genistein and daidzein).[37–39] In humans, 30–40% of the population can convert daidzein to equol.[40,41] The amount of urinary equol excretion is correlated with the consumption of soy products[10,42] and a reduced risk of breast cancer.[43,44] In vitro experiments suggest that equol might be more estrogenic than daidzein.[4,35,41] The actions of estrogens, including those of the phytoestrogens such as daidzein (and its equol metabolite) and genistein, the principal phytoestrogens in soy, are mediated through the estrogen receptors (ERs), of which there are two subtypes, ERα and ERβ.[16] These proteins are ligand-regulated transcription factors, and their tissue distribution, biological functions, and response to structurally diverse ligands are quite different.[16,45–49] The phytoestrogens are often considered to be ERβ-selective ligands.[31,50] Equol is a chiral molecule, capable of existing in two enantiomeric forms with potentially different biological activities, with natural equol, being the *S*-(−) enantiomer.[7,26,27] The relative binding affinity and transcriptional potency of the equol enantiomers for the two ER subtypes have not been reported previously.

To facilitate studies on the receptor interactions and estrogenic activity of equol and its enantiomers, as well as experiments in animal models of human diseases, we have developed, as reported here, a convenient method to prepare (±)-equol in multigram quantities from commercially available isoflavone precursors. It is not, however, an enantioselective synthesis, although enantioselective syntheses have been described for both optical antipodes of equol dimethyl ether starting from substituted phenylacetic acids, using a stereoselective alkylation and cyclization strategy.[51] Rather than attempt to replicate this multistep procedure, we chose simply to resolve *R*- and *S*-equol by chiral LC. Using a chiral β-cyclodextrin column, we obtained an excellent separation that could be scaled up readily to produce multimilligram amounts of the pure equol stereoisomers. The identity of the eluted material was confirmed by LC-ES/MS/MS, and the assignment of stereoisomers was done by CD; the first eluting peak gave a CD spectrum having a long-wavelength peak with a negative ellipticity, which is known to correspond to *S*-equol, the naturally occurring enantiomer, and the second peak, with a positive CD corresponding to *R*-equol.[26,27]

The two equol enantiomers showed very different behavior in terms of their binding affinities and transcriptional potencies with ERα and ERβ. In competitive binding affinity assays, we found that the binding affinity of the natural enantiomer, *S*-equol, is quite high and shows a 13-fold preference for ERβ, being similar in these respects to genistein (β/α = 180), the most estrogenic soy isoflavone, whereas *R*-equol shows a preference for ERα (β/α = 0.29). Daidzein, the isoflavone precursor of equol, binds more weakly and shows little ER subtype selectivity. In cotransfection transcription assays, the potency of the equol stereoisomers and of genistein nicely reflect their binding affinities for ERα and ERβ, although the strong ERβ preference of *S*-equol in terms of binding affinity is considerably muted and the transcriptional potency of daidzein is higher than expected from its affinity. Thus, genistein is the only compound that stands out as having an accentuated potency for ERβ in transcription assays, whereas both *S*-equol and genistein have a preference for ERβ in terms of binding. Whether these differences in ER subtype selectivity between binding and transcription assays are due to cellular metabolism or differential interaction with cellular coregulators is an issue that needs to be evaluated further.[52–55]

It is of note that the range of ERα binding affinity values reported for soy phytoestrogens is remarkably wide, with values for genistein spanning nearly a 100-fold range (RBA values 0.010–0.88) and for daidzein an 8-fold range (RBA values 0.01–0.08).[56] The ERα binding affinity values we have obtained for both of these compounds (Table 1) are clearly within these ranges, but we note that it proved far more difficult to obtain consistent binding affinity values for these two compounds than for a very wide range of other estrogens that we have evaluated in the same binding assay over a period of more than 30 years. Furthermore, in our hands genistein has a higher binding affinity for the ER in a preparation from lamb uterus, giving an RBA value of 0.69, a value that is similar to that obtained in rat uterine ER by Sheehan (RBA 0.45).[57] One would expect the ER from these uterine sources to be predominantly ERα.[16,58]

The different tissue distribution and distinct biological roles of ERα and ERβ and their potential for mediating divergent effects of estrogens on target tissue stimulation and proliferation highlight the importance of characterizing carefully the efficacy and potency of various estrogens through these two receptors. ERα is found in most estrogen target tissues, in some cases nearly by itself or accompanied by lower levels of ERβ. ERβ, on the other hand, is found by itself or in higher levels than ERα only in a few regions of the brain, in the ovary, prostate and testis.[16,58,59]

Although at this point not fully understood, the biological functions of ERα and ERβ have been studied both by the generation of gene knockout mice[60,61] and by the use of ER subtype-selective ligands.[62] One interesting hypothesis that has been advanced, which is consistent with the generally greater transcriptional effectiveness of ERα vs ERβ, is that estrogens acting through ERα effect a strong stimulatory or proliferative drive in target tissues (or estrogen-responsive tumors) and that estrogens acting through ERβ moderate this drive.[63] Thus, both the relative levels of ERα and ERβ, as well as the potency or efficacy selectivity of the particular estrogen that is acting through these two subtypes, could determine the ultimate magnitude of the stimulatory effect. Consistent with this hypothesis are the reports that as breast and prostate tumors become progressively more malignant, there is a general increase in the ratio of ERα to ERβ,[63–65] as well as the reports of hyperplasia in certain target tissues in ERβ knockout mice,[63] although the observations of all laboratories are not consistent on this latter issue.[66] In light of our study on the effects of equol and its enantiomers on the two estrogen receptor subtypes, it would appear prudent to evaluate carefully, in vivo, the biological effects of not only the isoflavones but also their metabolites and their stereoisomers. Such investigations would greatly help in evaluating the potential effects of the ingestion of soy isoflavones on human health and disease.

4. Conclusion

We have developed a simple synthetic route to equol from readily available isoflavanoid starting materials. Chiral HPLC provided ready access to the two enantiomers of equol and allowed us to examine, for the first time, their differential effects on the two estrogen receptor subtypes. S-equol shows a strong binding preference in favor of ERβ that is greater than that of its daidzein precursor and comparable to that of genistein, whereas the R isomer exhibits a binding selectivity for ERα. In vitro transfection assays show that the S isomer, however, has little ERβ selectivity in terms of transcriptional potency.

5. Experimental

5.1. Materials

Formononetin was a gift from Dr. Muralee Nair, Michigan State University, East Lansing, MI). Daidzein was purchased from Indofine Chemical (Belle Mead, NJ). Ammonium formate, ethanethiol and 20% $Pd(OH)_2$ on carbon were purchased from Aldrich Chemical Company.

5.2. Chemical synthesis, separation and spectroscopic methods

5.2.1. Reduction of formononetin. Formononetin (5.0 g, 18.6 mmol) was suspended in acetic acid (25 mL) together with ammonium formate (11.75 g, 186 mmol) and 20% $Pd(OH)_2$ on carbon (4.8 g). The reaction mixture was then refluxed for 1 h and allowed to cool to room temperature. After diluting with 50 mL of EtOAc, the reaction mixture was filtered through a 2 cm pad of Celite that was subsequently washed with an additional 150 mL of EtOAc. The filtrate was evaporated in vacuo to give a light brown oily residue which was purified on silica gel using 15% EtOAc/hexane as the solvent. Trituration of this oil with 125 mL of water gave a white fluffy solid that was filtered and dried to give 3.2 g (68%) of monomethyl equol. ^{1}H NMR (500 MHz, acetone-d_6): δ 7.05 (d, 8.4 Hz, 2H), 6.84 (d, 8.1 Hz, 1H), 6.74 (d, 8.4 Hz, 2H), 6.30 (dd, 2.36 Hz, 8.1 Hz, 1H), 6.23 (d, 2.36 Hz, 1H), 4.15 (m, 1H), 3.87 (t, 10.72 Hz, 1H), 3.67 (s, 3H), 3.00 (m, 1H), 2.81 (m, 2H). Mp 156–158 °C; reported:[67] 158 °C.

5.2.2. Synthesis of (±)-equol from monomethyl equol. Aluminum trichloride (2.10 g, 15.7 mmol) was mixed with ethane thiol (12 mL) together with 5 mL of anhydrous CH_2Cl_2 at 0 °C under nitrogen. The light yellow solution was allowed to stir for 5 min. after which monomethyl equol (1.0 g, 3.9 mmol) was added in one portion. After stirring for 1 h at 0 °C, the reaction was quenched by addition of water and the mixture was warmed to 40 °C and left to stir at that temperature, with the flask open to air, until all traces of ethane thiol were completely removed. A white solid which precipitated out was dissolved in EtOAc (20 mL), washed with 10% HCl and dried on $MgSO_4$. The organic solvent was removed in vacuo to give a light brown oil which was chromatographed on silica gel using 35% EtOAc/hexane as the eluent to give 0.78g (84%) of (±)-equol. ^{1}H NMR (500 MHz, acetone-d_6): δ 6.93 (d, 8.58 Hz, 2H), 6.75 (d, 8.15 Hz, 1H), 6.69 (d, 8.58 Hz, 2H), 6.27 (dd, 2.36 Hz, 8.15 Hz, 1H), 6.22(d, 2.36 Hz, 1H), 4.11 (m, 1H), 3.79 (t, 10.72 Hz, 1H), 2.97 (m, 1H), 2.75 (m, 2H). ^{13}C NMR (125 MHz, acetone-d_6): δ 155.8, 154.5, 132.1, 129.8, 127.9, 115.2, 113.1, 107.8, 102.7, 70.7, 37.5, 31.5. Mp 184–187 °C; reported[7] 187–189 °C.

5.2.3. Conversion of daidzein to (±)-equol. Daidzein (5.0 g, 18.6 mmol) was suspended in acetic acid (25 mL) together with ammonium formate (11.75 g, 186 mmol) and 20% $Pd(OH)_2$ on carbon (4.8 g). The reaction mixture was then refluxed for 1 h and allowed to cool to room temperature. After diluting with 50 mL of EtOAc, the reaction mixture was filtered through a 2 cm pad of Celite that was subsequently washed with an additional 150 mL of EtOAc. The filtrate was evaporated in vacuo to give a light brown oily residue that was purified on silica gel using 30% EtOAc/hexane as the solvent. Trituration of this oil with 125 mL of water gave a white

fluffy solid which was filtered and dried to give 2.9 g (61%) of (±)-equol. ^{1}H NMR (500 MHz, acetone-d_6): δ 6.93 (d, 8.58 Hz, 2H), 6.75 (d, 8.15 Hz, 1H), 6.69 (d, 8.58 Hz, 2H), 6.27 (dd, 2.36 Hz, 8.15 Hz, 1H), 6.22(d, 2.36 Hz, 1H), 4.11 (m, 1H), 3.79 (t, 10.72 Hz, 1H), 2.97 (m, 1H), 2.75 (m, 2H). ^{13}C NMR (125 MHz, acetone-d_6): δ 155.8, 154.5, 132.1, 129.8, 127.9, 115.2, 113.1, 107.8, 102.7, 70.7, 37.5, 31.5. Mp 184–187 °C; reported:[7] 187–189 °C.

5.2.4. Resolution of equol enantiomers by chiral liquid chromatography (LC). Initial LC separation was performed using an HP1050 quaternary pumping system (Hewlett Packard, Palo Alto, CA) and a Dionex AS3500 autosampler (Dionex, Sunnyvale, CA) attached to a PC1000 data handling system (Thermo Separation Products, San Jose, CA). UV detection was provided by a Spectraphysics MWD (Thermo Separation Products, San Jose, CA) at a wavelength of 280 nm. Enantiomeric separation was achieved using a Cyclobond I *2000* RSP β-cyclodextrin-R,S-hydroxypropyl ether chiral stationary phase (analytical column 250×2.0 mm, 5 μm particle size; semi-preparative column 250×10 mm, 5 μm, particle size, Astec, NJ, USA) and an isocratic mobile phase consisting of 65% 0.1% formic acid (aq) and 35% of acetonitrile. Respective flow rates for the analytical and semi-preparative columns were 0.25 and 5 mL/min. Injection volumes ranged from 5–100 μL, and all separations were performed at ambient temperature.

5.2.5. Mass spectrometry. LC with tandem mass spectrometry (LC-MS/MS) was conducted using a Waters 2790 liquid handling system (Waters Assoc., Milford, MA) and a Quattro Ultima triple quadrupole mass spectrometer equipped with an electrospray (ES) interface (Micromass, Manchester, UK). The entire column effluent (0.25 mL/min) was directed into the ion source held at 120 °C for acquisition of positive ions in the product ion or multiple reaction monitoring modes (MRM), as previously described.[68]

5.2.6. Circular dichroism (CD) spectrophotometry. CD spectra of separated equol enantiomers were obtained at ambient temperature by using a Jasco 500A spectropolarimeter equipped with a quartz cell (1 cm path length) containing equol solutions in 50% aqueous methanol that produced 1 AU in a spectrophotometer. The spectropolarimeter was calibrated using (*1S*)-(+)-10-camphorsulfonic acid before use.

5.3. Estrogen receptor binding assays

Relative binding affinities were determined by competitive radiometric binding assays using 10 nM [^{3}H]E_2 as tracer, using methods previously described.[28,49,69] The source of ER was purified full-length human ERα and ERβ purchased from Pan Vera (Madison, WI).[28,49,69] Incubations were done at 0 °C for 18–24 h, and hydroxylapatite was used to absorb the purified receptor-ligand complexes (human ERs).[28,49,69] The binding affinities are expressed as relative binding affinity (RBA) values, where the RBA of estradiol is 100%; under these conditions, the K_d of estradiol for ERα is ca. 0.2 nM, and for ERβ 0.5 nM.[28] The determination of these RBA values is reproducible in separate experiments with a CV of 0.3, and the values shown represent the average ± range or SD of 2 or more separate determinations, respectively.

5.4. Cell culture and transcription activation assays

Human endometrial carcinoma (HEC-1) cells were maintained in culture as described.[30] Transfection of HEC-1 cells in 24-well plates used a mixture of 0.35 mL of serum-free Improved Minimal Essential medium (IMEM) and 0.15 mL of Hank's Balanced Salt Solution (HBSS) containing 5 μL of lipofectin (Life Technologies, Rockville, MD), 1.6 μg of transferrin (Sigma, St. Louis, MO), 0.5 μg of pCMVβ-galactosidase as internal control, 1 μg of the reporter gene plasmid, 100 ng of ER expression vector, and carrier DNA to a total of 3 μg DNA per well. The cells were incubated at 37 °C in a 5% CO_2 containing incubator for 6 h. The medium was then replaced with fresh medium containing 5% charcoal-dextran treated calf serum and the desired concentrations of ligands. Reporter gene activity was assayed at 24 h after ligand addition. Luciferase activity, normalized for the internal control β-galactosidase activity, was assayed as described.[30]

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